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Matrix-M adjuvant enhances antibody, cellular and protective immune responses of a Zaire Ebola/Makona virus glycoprotein (GP) nanoparticle vaccine in mice

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ABSTRACT

Ebola virus (EBOV) causes severe hemorrhagic fever for which there is no approved treatment or preventive vaccine. Immunological correlates of protective immunity against EBOV disease are not well understood. However, non-human primate studies have associated protection of experimental vaccines with binding and neutralizing antibodies to the EBOV glycoprotein (GP) as well as EBOV GP-specific $CD4^+$ and $CD8^+$ T cells. In this report a full length, unmodified Zaire EBOV GP gene from the 2014 EBOV Makona strain (EBOV/Mak) was cloned into a baculovirus vector. Recombinant EBOV/Mak GP was produced in Sf9 insect cells as glycosylated trimers and, when purified, formed spherical 30–40 nm particles. In mice, EBOV/Mak GP co-administered with the saponin adjuvant Matrix-M was significantly more immunogenic, as measured by virus neutralization titers and anti-EBOV/Mak GP IgG as compared to immunization with $AlPO_4$ adjuvanted or non-adjuvanted EBOV/Mak GP. Similarly, antigen specific T cells secreting IFN-γ were induced most prominently by EBOV/Mak GP with Matrix-M. Matrix-M also enhanced the frequency of antigen-specific germinal center B cells and follicular helper T (T_{FH}) cells in the spleen in a dose-dependent manner. Immunization with EBOV/Mak GP with Matrix-M was 100% protective in a lethal viral challenge murine model; whereas no protection was observed with the $AlPO_4$ adjuvant and only 10% (1/10) mice were protected in the EBOV/Mak GP antigen alone group. Matrix-M adjuvanted vaccine induced a rapid onset of specific IgG and neutralizing antibodies, increased frequency of multifunctional CD4+ and $CD8^+$ T cells, specific T_{FH} cells, germinal center B cells, and persistence of EBOV GP-specific plasma B cells in the bone marrow. Taken together, the addition of Matrix-M adjuvant to the EBOV/Mak GP nanoparticles enhanced both B and T-cell immune stimulation which may be critical for an Ebola subunit vaccine with broad and long lasting protective immunity.

1. Introduction

The extent of the recent Ebola outbreak in Western Africa has brought world-wide attention to the lack of therapeutic and preventive measures against EBOV disease (EVD) and has spurred intense actions towards development of vaccine candidates, and a broad range of technologies and approaches are being explored [1,2].

Matrix-M™, an adjuvant based on saponin extracted from the *Quillaja saponaria* Molina tree induces high and long-lasting levels of broadly reacting antibodies supported by a balanced TH1 and TH2 type of response, including biologically active antibody isotypes such as murine IgG2a, multifunctional T cells and cytotoxic T lymphocytes [3–10]. The mode-of-action of Matrix-M adjuvant has not been elucidated in detail; however, the adjuvant promotes

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rapid and profound effects on cellular drainage to local lymph nodes creating a milieu of activated cells including T cells, B cells, Natural Killer cells, neutrophils, monocytes and dendritic cells [10–12].

Matrix-M has shown potent adjuvant activities, inducing enhanced immune responses in preclinical [3,4,6,7,13] and human clinical studies [5], most recently with H7N9 avian influenza virus like particle vaccine where Matrix-M adjuvanted vaccine had a significant dose-sparing effect and an acceptable safety profile [14].

Immune correlates of durable protection to EVD are to date not clearly identified. Preclinical immunization studies in rodents and non-human primates (NHP), as well as protective efficacy demonstrated with reconvalescent plasma [15] and monoclonal antibody cocktails *in vitro*, in rodents [16], in NHP [17–19] and in man [20], point towards antibodies being a crucial protective element.

The potential importance of a cellular response supporting the development of a protective humoral response was demonstrated by Marzi and co-workers [21] in a NHP challenge study with a live vector based rVSV/ZEBOV-GP vaccine. T cell depletion studies showed $CD4^+$ T cells to be crucial during the generation of the antibody response but not at challenge, implicating a strong supportive role of $CD4^+$ cells in the development of a protective antibody response.

The role of prophylactic effector T cell responses to protect against EVD is unclear; however, published data show a possible supportive role in the generation of protection in NHP challenge studies [22]. Particularly, IFN-γ producing $CD4^+$ cells are responsible for a whole range of effector functions and polarization of both humoral and cellular responses [23,24]. In addition, studies evaluating chimpanzee adenovirus EBOV GP vectors suggest a role of $CD8^+$ T cell mediated responses in protection [22].

To date, the majority of EBOV vaccine approaches with positive NHP protection data involve the use of viral vectors or viral chimaeras [2]. Inactivated virus and sub-unit approaches have had limited success, particularly in non-human primates [2,25]. However, there are some positive studies using potent TH1 type stimulatory adjuvants [26,27].

In the present study, we used EBOV/Mak GP nanoparticles produced in Sf9 cells infected with recombinant baculovirus expressing EBOV/Mak GP [28] combined with Matrix-M [12]. Mice were immunized with EBOV/Mak GP alone or adjuvanted with Matrix-M or $AlPO_4$. The EBOV/Mak GP specific IgG, IgG1 and IgG2a response, as well as the virus neutralizing antibody response and protection against viral challenge were evaluated.

In a separate series of experiments, the nature of the T cell responses were analysed by cytokine profiling and intracellular staining. In addition, GC reaction and T_{FH} cell frequencies were measured, and the longevity of T and B cell responses studied at Day 60 after immunization.

2. Materials and methods

2.1. EBOV glycoprotein nanoparticles

The wild-type full-length, unmodified Zaire EBOV glycoprotein (GP) gene from the 2014 Makona EBOV was cloned into recombinant baculovirus and expressed in *Spodoptera frugiperda* Sf9 insect cells as described by Hahn et al. [28]. Purified EBOV Makona GP (EBOV/Mak GP) nanoparticles are composed of multiple GP trimers assembled into spherical particles 36 ± 4 nm (as measured by dynamic light scattering). Recombinant GP nanoparticles have a core region which contains the glycoprotein 2 (GP2) 'fusion subunits' with 2–9 or more "chalice-like" glycoprotein 1 (GP1) trimers 'attachment subunits' extending outward.

2.2. Matrix-M adjuvant

Novavax proprietary adjuvant Matrix-M™ is a saponin-based adjuvant consisting of two populations of individually formed 40 nm sized Matrix particles, each with a different and well-characterized saponin fraction with complementary properties (Fraction-A and Fraction-C, respectively). Matrix-M, used in this study, consists of 85% Matrix-A and 15% Matrix-C. The Matrix particles are formed by formulating purified saponin from *Q. saponaria* Molina with cholesterol and phospholipid [29].

2.3. Animals and ethics statement

All animal research was conducted in accordance with the NRC Guide for the Care and Use of Laboratory Animals, the Animal Welfare Act and the CDC/NIH Biosafety in Microbiological and Biomedical Laboratories.

Following EBOV challenge, mice were monitored two or more times daily and their status evaluated by direct observation. Animal were assigned a disease score based on assessments given for weight loss, dyspnea, responsiveness, abnormal hair coat, eye and nasal abnormalities including discharge (Supplemental Tables 1 and 2). Mice that achieved a disease score of 12 were considered moribund, indicating that euthanasia criteria were met and mice were euthanized for humane purposes by CO_2 inhalation followed by confirmatory cervical dislocation.

2.4. Vaccination and challenge

BALB/c mice (6–8 weeks old; Harlan Laboratories Inc., Frederick, MD) were immunized by SC injection at Days 0, 14 and 28 with 5 μg EBOV/Mak GP antigen alone or adjuvanted with $AlPO_4$ (50 μg) or Matrix-M (5 μg) in a dose volume of 100 μl. Blood for serum was collected via the retro-orbital route on Days 0, 21 and 28. Prior to blood collection, animals were anesthetized with isoflurane. On Study Day 32, the mice were transferred to the BSL-4 facility (Texas Biomedical Research Institute, San Antonio, TX) and on Study Day 42 (14 days following the third immunization), mice were challenged by an intraperitoneal inoculation of 1000 pfu mouse adapted Zaire EBOV strain 1976 Mayinga [30].

2.5. Vaccination and characterization of responses

BALB/c mice ($n = 5$ per group) were immunized by IM administration (50 μl injection volume) at Days 0 and 21 with EBOV/Mak GP alone or mixed with $AlPO_4$ (50 μg) or Matrix-M adjuvant (2.5 or 5 μg). Blood samples were collected via the retro-orbital route at Days 0, 14, 21, 28 and 60. Spleen and bone marrow samples were collected at Days 28 and 60. Spleen and bone marrow samples were suspended in PBS containing 2% fetal bovine serum (FBS) for further preparation.

2.6. Virus neutralizing activity

Serum samples from day 28 were evaluated for anti-EBOV/Mak neutralizing antibody responses at U.S. Army Medical Research Institute of Infectious Diseases, Fredrick, MD using a pseudovirion neutralization assay [31]. For this assay, the vesicular stomatitis virus G protein was removed and replaced with luciferase reporter. This VSV luciferase expressing core was pseudotyped using the plasmid pWRG/EBOV-Z76(opt) that expresses the Zaire EBOV 1976 (Mayinga) GP. PsVs were prepared in 293 T cells. The assay positive control was serum from a rabbit vaccinated three times with the pWRG/EBOV-Z76(opt), a Zaire EBOV 1976 Mayinga GP DNA vaccine.

2.7. Anti-EBOV/Mak GP ELISA

EBOV/Mak GP specific serum antibodies were quantitated by enzyme linked immunosorbent assay (ELISA) as described previously [32]. NUNC MaxiSorp microtiter plates were coated with 2 μg/ml of EBOV/Mak GP (Novavax) overnight at 2–8 °C. Mouse anti-EBOV/Mak GP monoclonal antibody 4F3 from IBT Bioservices (Gaithersburg, MD) was used as the positive control.

2.8. IFN-γ ELISPOT assay

Spleen cells were washed twice with PBS containing 2% FBS and counted. IFN-γ ELISPOT assays were performed using mouse IFN-γ ELISPOT kits (eBioscience, San Diego, CA) according to the manufacturer's procedure. Briefly, anti-IFN-γ antibody (15 μg/ml in PBS) was used to coat ELISPOT plates (Millipore, Darmstadt, Germany) and incubated, overnight at 4 °C. The plates were washed with PBS and blocked with RPMI1640 medium plus 5% FBS for 1–2 h at room temperature. A total of 3×10^5 splenocytes in a volume of 200 μl were stimulated with pools of 15-mer EBOV GP peptides with 11 overlapping amino acids (2.5 μg/ml) covering the entire EBOV GP sequence. Phorbol myristic acetate (PMA) (50 ng/ml) plus ionomycin (200 ng/ml) was used as positive control and medium as negative control. Each stimulation condition was carried out in triplicate. Assay plates were incubated overnight at 37 °C in a 5% CO_2 incubator and developed using BD ELISPOT AEC substrate set (BD Biosciences, San Diego, CA). Spots were counted and analyzed using an ELISPOT reader and Immunospot software (Cellular Technology, Ltd., Shaker Heights, OH). The number of IFNγ-secreting cells induced by EBOV GP peptides was obtained by subtracting the background number in the medium controls from the GP-peptide stimulated wells. Data shown in the graph are the average of triplicate wells.

2.9. Anti-EBOV/Mak GP IgG secreting cell ELISPOT

To measure GP-specific IgG-secreting cells, ELISPOT plates were coated with EBOV/Mak GP (2.5 μg/ml) and incubated overnight at 4 °C. Plates were washed and blocked as described above. Triplicates of 3-5 $\times 10^5$ splenocytes or bone marrow cells per well were plated and incubated overnight at 37 °C. Plates were washed and goat anti-mouse IgG-HRP was added and incubated for 1.5 h. Spots were developed and counted as described above. The average spot number from triplicate wells were calculated and presented.

2.10. Surface staining for cell phenotypes and intracellular staining for cytokines

For surface staining, cells were first incubated with anti-CD16/32 antibody to block the Fc receptor. To characterize the germinal center cells, 1×10^6 splenocytes were incubated at 4 °C for 30 min with a mixture of the following antibodies: B220-PerCP, CD19-APC, GL7-BV421, CD95-PE-Cy7 (BD Biosciences) and the yellow LIVE/DEAD® dye (Life Technologies, NY). To stain T follicular helper cells, 1×10^6 splenocytes were incubated with CXCR5-Biotin, washed, then incubated with a mixture of antibodies including CD3-BV650, B220-PerCP, CD4-PE-Cy7, Streptavidin-BV421, PD-1-APC, CD69-FITC and CD49b-PE (BD Biosciences) and the yellow LIVE/DEAD® dye. Cells were washed and suspended in PBS containing 2% FBS for analysis.

For intracellular staining for cytokines, splenocytes were cultured in a 96-well U-bottom plate at 1×10^6 cells per well. The peptide stimulation was performed as described for the ELISPOT cultures. The plate was incubated 6 h at 37 °C in the presence of BD GolgiPlug™ and BD GolgiStop™ (BD Biosciences). Cells were washed and incubated for 20 min at 4 °C with a mixture of antibodies including CD3-BV650, CD4-PerCP, CD8-FITC, CD44-APC-Cy7 and CD62L-PE-Cy7 (BD Pharmingen, CA) and the yellow LIVE/DEAD® dye (Life Technologies, NY). After two washes, cells were fixed with Cytofix/Cytoperm (BD Biosciences) for 30 min at 4 °C, followed by two washes with BD Perm/Wash™ (BD Biosciences). Cells were incubated with antibodies to IFN-γ-APC, IL-2-BV 421 and TNFα-PE (BD Biosciences) overnight at 4 °C. The cells were washed and re-suspended in 1xBD Perm/Wash buffer for data acquisition. All staining samples were acquired using a LSR-Fortessa flow cytometer (Becton Dickinson, San Jose, CA) and the data were analysed with Flowjo software version Xv10 (Tree Star Inc., Ashland, OR).

2.11. Statistical analysis

Statistical analysis was performed using SAS software version 9.4. Pairwise comparisons with Tukey's adjustment from ANOVA used group as the independent variable and log-transformed titer result as the dependent variable to determine significance between groups. Statistical analysis of Kaplan-Meier survival curves was done by Log-Rank analysis.

3. Results

3.1. Immunogenicity and protective efficacy of EBOV/Mak GP vaccine

The immunogenicity of the EBOV/Mak GP nanoparticle vaccine was evaluated with and without adjuvant in a mouse model. Mice were vaccinated on Days 0, 14 and 28 by SC injection with 5 μg of EBOV/Mak GP alone or formulated in Matrix-M or $AlPO_4$ adjuvant. Analysis of sera obtained on Day 28 indicated that the Matrix-M adjuvanted EBOV/Mak GP induced high levels of antigen-specific IgG antibodies (Fig. 1A). The response obtained following immunization with EBOV/Mak GP with Matrix-M was significantly higher than those induced by EBOV/Mak GP alone ($p < 0.0001$) or EBOV/Mak GP adjuvanted with $AlPO_4$ ($p < 0.0001$).

Low levels of neutralization activity were observed in sera from mice immunized with the EBOV/Mak GP alone and EBOV/Mak GP adjuvanted with $AlPO_4$ (Fig. 1B). Neutralization titers observed in sera from mice immunized with EBOV/Mak GP with Matrix-M were thirty-two-fold higher than that obtained with EBOV/Mak GP alone ($p < 0.0001$). As PsVs expressing the EBOV 1976 Mayinga strain GP were used, assay is measuring the cross-neutralizing activity of anti-EBOV/Mak GP against the Mayinga GP.

On Day 42, mice were challenged by an intraperitoneal inoculation of 1000 pfu mouse adapted Zaire EBOV strain 1976 Mayinga. Control mice started to succumb to infection after three days while mice vaccinated with EBOV/Mak GP alone or EBOB/Mak GP adjuvanted with $AlPO_4$ succumbed at day five or six, respectively. Twenty-one days after challenge infection, all mice vaccinated with Matrix-M adjuvanted EBOV/Mak GP and one mouse vaccinated with EBOV/Mak GP alone were alive and healthy (Fig. 1C).

3.2. $CD4^+$, $CD8^+$, and multifunctional T cell response

To assess T cell responses, the number of IFN-γ secreting T cells after *ex vivo* stimulation of spleen cells with EBOV/Mak GP peptides in an ELISPOT assay were determined. At Day 28, IFN-γ secreting cells increased in a Matrix-M dose-dependent manner in spleens from mice immunized with EBOV/Mak GP with Matrix-M (Fig. 2A and B). The average number of IFN-γ-secreting cells in groups receiving EBOV/Mak GP with 5.0 and 2.5 μg of Matrix-M were 17- and 10-fold higher, respectively, than in the group receiving EBOV/Mak GP alone and 8- and 5-fold higher, respectively, than in the group receiving EBOV/Mak GP with $AlPO_4$ (Fig. 2A).

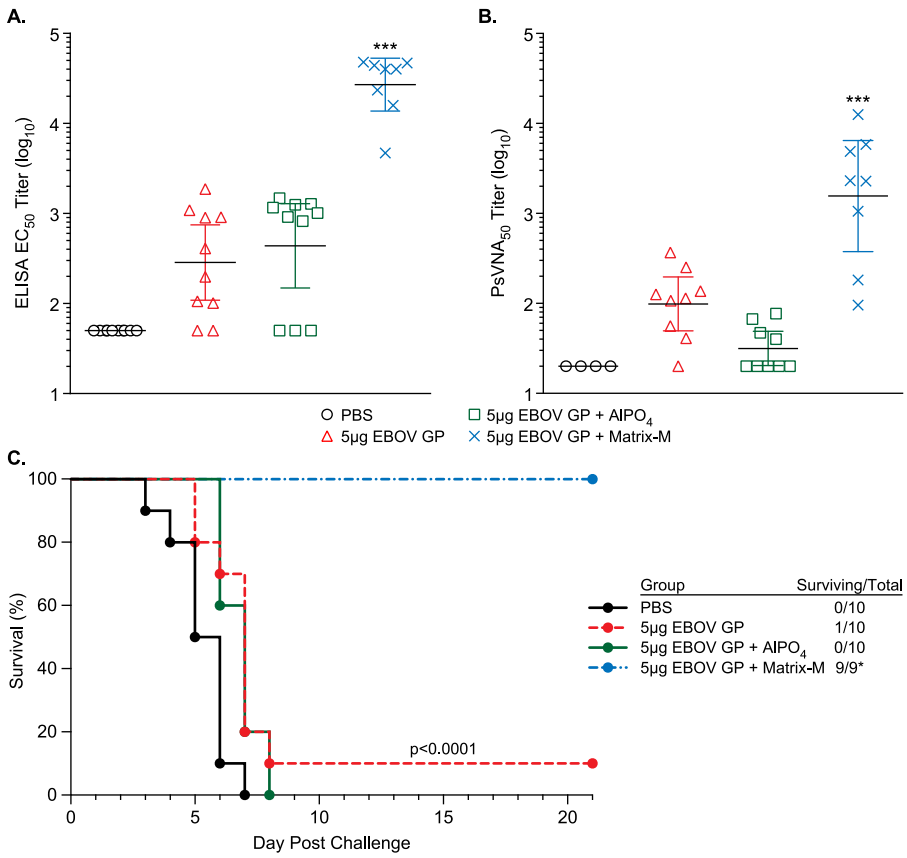

Fig. 1. EBOV/Mak GP induced antibody response and protective efficacy. Mice were immunized SC on Days 0, 14 and 28 with 5 μg EBOV/Mak GP, 5 μg EBOV GP adjuvanted with 50 μg $AlPO_4$ or 5 μg EBOV/Mak GP adjuvanted with 5 μg Matrix-M. Serum was obtained on Day 28 and evaluated by ELISA for anti-EBOV/Mak GP IgG (A) or anti-Ebola virus neutralizing antibody (B). Black bars represent the group GMT and error bars indicate 95% confidence intervals of the GMT, *** $p < 0.0001$. On Day 42, mice were infected with 1000 pfu mouse adapted Zaire EBOV strain 1976 Mayinga. Following challenge, mice were observed daily for morbidity and mortality for a period of 21 days. (C) Kaplan-Meier survival curve for infected mice. Statistical analysis of Kaplan-Meier survival curve was done by Log-Rank analysis.

By Day 60, the number of IFN-γ secreting cells in spleens from mice immunized with EBOV/Mak GP with 5 μg of Matrix-M was still 12-fold higher than observed in mice immunized with EBOV/Mak GP alone and 3-fold higher than in mice immunized with EBOV/Mak GP with $AlPO_4$ (Fig. 2B). The increased numbers of IFN-γ secreting cells in mice immunized with EBOV/Mak GP with 2.5 μg Matrix-M were also maintained at Day 60 but at a lower level than with 5 μg Matrix-M.

We further assessed Matrix-M induced $CD4^+$ and $CD8^+$ T cell responses by intracellular staining of cytokines combined with cell surface markers. Analysis of splenocytes by flowcytometric staining at Day 28 showed that both $CD4^+$ and $CD8^+$ T cells from EBOV/Mak GP with Matrix-M groups secreted IFN-γ, TNFα and IL-2 (Fig. 2C–E). The frequency of cytokine-secreting $CD4^+$ and $CD8^+$ T cells was much higher in spleens from the EBOV/Mak GP with Matrix-M groups than the responses observed in control mice (PBS or Matrix-M alone), mice receiving EBOV/Mak GP alone or EBOV/Mak GP with $AlPO_4$ (Fig. 2C–E). The frequency of T cells that simultaneously produce two or three cytokines was also evaluated at Day 28. Both $CD4^+$ and $CD8^+$ T cells producing either two or three cytokines were detected at marked levels only in spleens from the mice immunized with EBOV/Mak GP with Matrix-M.

3.3. Germinal center and T follicular helper cell responses

The frequency and absolute number of GC B cells in the spleen were analysed by flowcytometric staining (Fig. 3A). The analysis showed that at Day 28, EBOV/Mak GP adjuvanted with 2.5 and 5 μg of Matrix-M induced responses with a GC frequency of 1.99 ± 0.80% and 2.83 ± 1.63%, respectively, in comparison to placebo, Matrix-M control, EBOV/Mak GP alone or EBOV/Mak GP with $AlPO_4$ (0.51 ± 0.09, 0.72 ± 0.25, 0.66 ± 0.13 and 0.75 ± 0.51%, respectively) (Fig. 3B). Accordingly, the absolute GC cell number in the spleen also increased in the groups receiving Matrix-M (Fig. 3C). By Day 60, the frequency and absolute number returned to background level (Fig. 3D and E).

Analysis of T_{FH} cells frequencies at Day 28 showed that EBOV/Mak GP with 2.5 or 5 μg of Matrix-M induced higher frequencies of T_{FH} cells than the EBOV/Mak GP alone or with $AlPO_4$ (Fig. 4A and B). Accordingly, the absolute number of T_{FH} cells was also enhanced by EBOV/Mak GP with Matrix-M compared to EBOV/Mak GP alone or with $AlPO_4$ (Fig. 4C). By Day 60, the frequency and absolute number of T_{FH} cells retracted to near background levels (Fig. 4D and E).

3.4. Persistence of EBOV/Mak GP-specific plasma cells in bone marrow

In order to assess the influence of Matrix-M on the formation of EBOV/Mak GP-specific plasma cells, the number of IgG-producing cells in spleen and bone marrow was analysed in ELISPOT assays at Day 60 after immunization. The analysis at Day 60 demonstrated only a few EBOV/Mak GP-specific IgG-secreting cells (<6/10^6 splenocytes) in the spleens from mice immunized with Matrix-M adjuvanted EBOV/Mak GP vaccine (Fig. 5A). No IgG-secreting cells

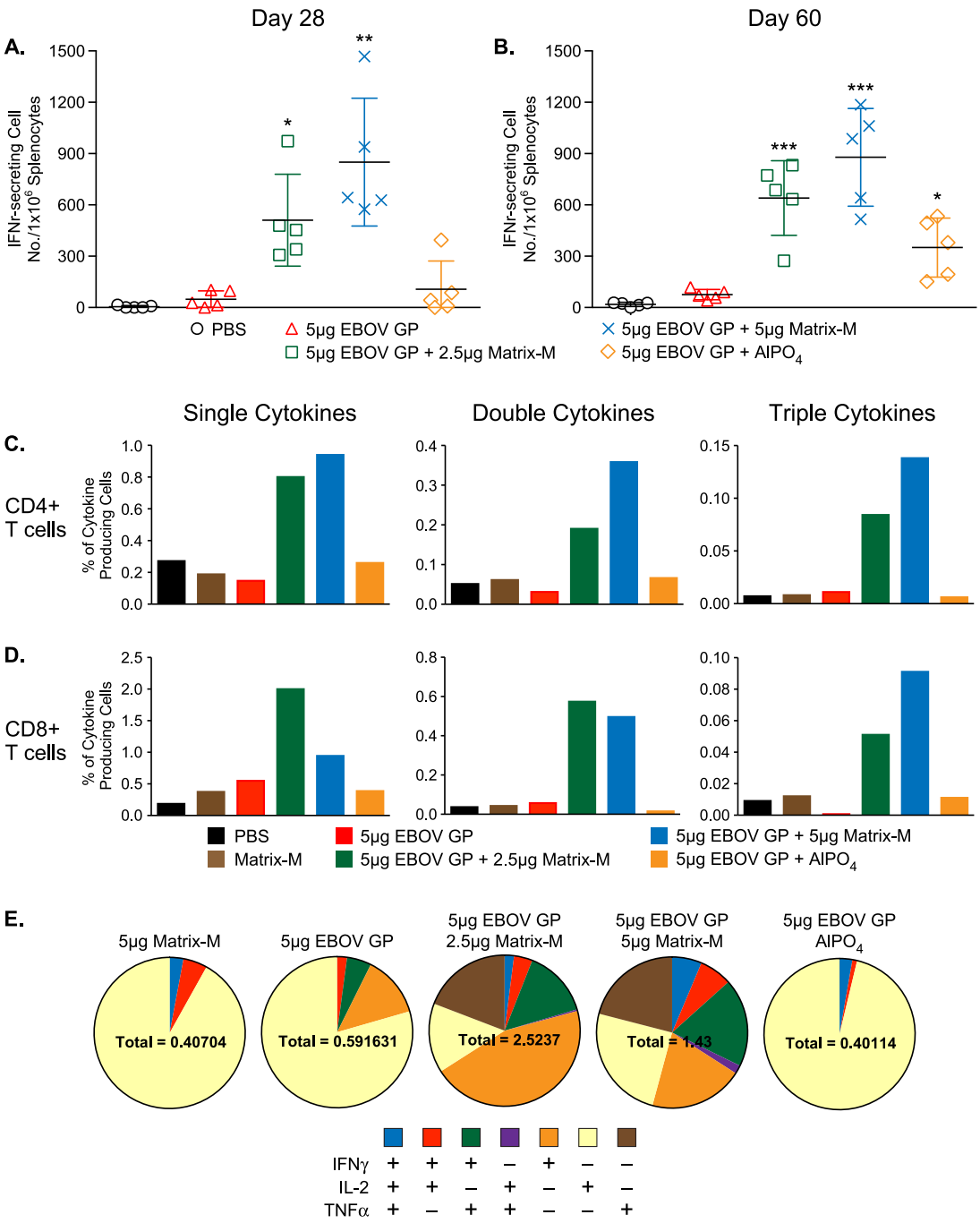

Fig. 2. Matrix-M enhanced CD4⁺ T cell and CD8⁺ T cell responses and multifunctional T cell response to EBOV/Mak GP. Spleen cells were stimulated with EBOV/Mak GP peptide pools covering the entire GP sequence. Culture medium or PMA (50 ng/ml) plus ionomycine (200 ng/ml) were used as negative and positive controls. IFN-γ positive spots from Day 28 (A) and 60 (B) were counted and analyzed with an ELISPOT reader and associated software. Background numbers of the medium controls were subtracted from the numbers of peptides-stimulated wells and a mean was derived from the triplicates. Cells from all five mice in the same group at Day 28 were pooled and incubated with either medium alone, or GP peptide pools, or PMA plus ionomycin for 6 h at 37 °C with the presence of BD Golgi-stop/Golgi-plug. Cells were then harvested and stained for cell surface markers and intracellular cytokines. Frequency of cytokines was analyzed using Flowjo software and Flowjo Boolean function by gating on live $CD3^+CD44^+CD62L^-CD4^+$ effector memory T cells or live $CD3^+CD44^+CD62L^-CD8^+$ effector memory T cells. The sum for single cytokines, double cytokines or triple cytokines represent the sum of the frequency of cells expressing any one of the three cytokines (IFN-γ, TNFα and IL-2), any two of the three cytokines or all three cytokines. The proportion of cells expressing any combination of cytokines for $CD8^+$ T cells is shown in the pie chart (D). The result is representative of two separate experiments. Black bars indicate group means and error bars represent standard deviation, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

were detected in the spleens from mice immunized with EBOV/Mak GP alone and EBOV/Mak GP with $AlPO_4$ (Fig. 5A). In contrast, high numbers of EBOV/Mak GP-specific IgG-secreting cells appeared in bone marrow from mice that received Matrix-M adjuvanted EBOV/Mak GP (Fig. 5B), demonstrating residency of EBOV/Mak GP specific plasma cells in the bone marrow.

4. Discussion

For reasons not fully understood, the EBOV surface glycoprotein has proven to be inherently poorly immunogenic requiring either genetic vectors or strong adjuvant to induce substantial and protective immune responses. Due to these factors we explored the

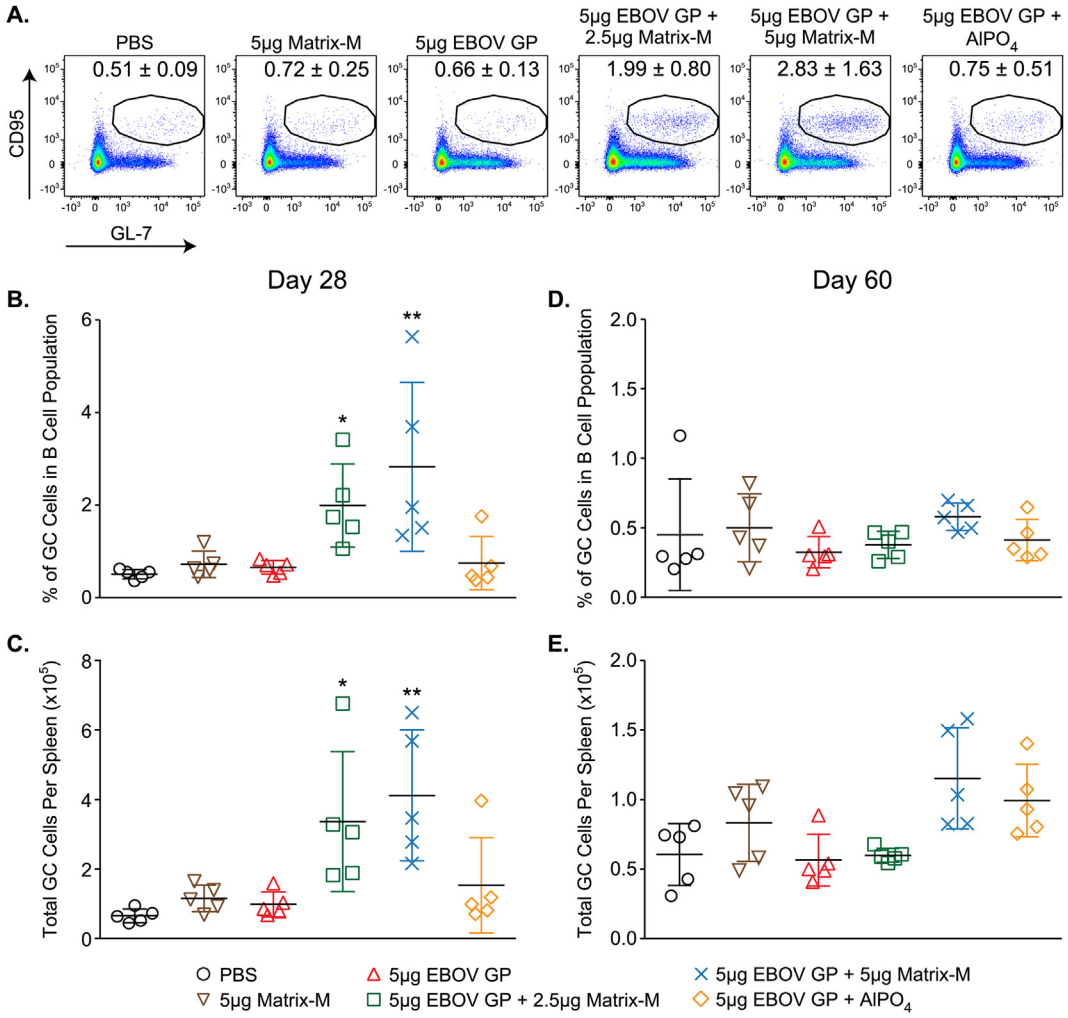

Fig. 3. Matrix-M enhanced GC cell response. Fresh splenocytes were stained for GC B cells and data was acquired as described in Materials and Methods. Data was analyzed with Flowjo software. Dead cells were excluded from analysis with Invitrogen LIVE/DEAD™ fixable yellow dye. (A), GC cells were defined as $CD95^+GL\text{-}7^+$ on $B220^+$ B cell gate and the numbers in the dot-plot of representative mice indicate the mean and standard deviation of GC frequency from all five mice in the same group at Day 28. GC cell frequencies from individual mice are shown for Days 28 (B) and Day 60 (D). The absolute GC cell number per spleen from Days 28 (C) and 60 (E) was calculated by multiplying the frequency of GC cells with the total number of splenocytes in the spleen. Black bars indicate group means and error bars represent standard deviation, * $p < 0.05$, ** $p < 0.01$.

use of the saponin adjuvant, Matrix-M, for the development of a protective, protein-based vaccine against EBOV disease.

In the present study, a recombinant EBOV/Mak full length GP nanoparticle vaccine formulated with Matrix-M, $AlPO_4$ or saline was evaluated. Immunization of mice with non-adjuvanted or $AlPO_4$ adjuvanted EBOV/Mak GP induced modest antibody and cellular responses. The inability of $AlPO_4$ to enhance the production of anti-EBOV/Mak GP IgG was unexpected. One possible explanation is that the structure of the GP nanoparticle is critical for an optimal antibody response and absorption of the nanoparticle onto $AlPO_4$ altered the nanoparticle structure.

When adjuvanted with Matrix-M, purified EBOV/Mak GP nanoparticles were highly immunogenic and protective in a murine challenge model. Immunization of mice with Matrix-M adjuvanted EBOV/Mak GP resulted in a significant increase in anti-EBOV/Mak GP IgG and EBOV neutralizing antibody. In addition, Matrix-M adjuvanted EBOV/Mak GP conferred 100% protection from a lethal EBOV challenge.

Co-administration of the EBOV/Mak GP with Matrix-M induced the production of a balanced IgG1 and IgG2a subclass response (see Supplemental Data). In the absence of adjuvant or with $AlPO_4$, minimal IgG2a antibody was detected. Blaney et al. [33], showed in a rabies/EBOV chimaera vaccine model in non-human primates (NHP) that the antibody isotype played a role in virus neutralization and protection against EBOV challenge. Murine IgG2a antibody is the equivalent of human IgG1 antibody that binds efficiently to IgG-Fc receptors (FcγR) and complement (C1q) [34,35] and may help resolving viral infections e.g., through antibody-dependent cell-mediated cytotoxicity. The benefits of IgG class switch to murine IgG2a isotype was reported by Wilson and co-workers [36] who raised a panel of monoclonal antibodies to the EBOV glycoprotein. All antibodies that were completely protective *in vivo* were of the IgG2a subclass.

Matrix-M adjuvant has been evaluated in numerous preclinical studies [4,6–12] without any reports of cytotoxicity or side effects. In clinical trials the use of Matrix-M has been reported as well tolerated and was demonstrated to have dose sparing properties [5]. However, Matrix-M adjuvanticity has been associated with a potent induction of leukocyte activation and migration into the draining lymph nodes [10–12]. Even though there is no evidence of pattern-recognition receptor stimulation by Matrix-M, there is a strong activation of T cells [3,5–7,10]. The presence of $CD8^+$ T cells secreting IFN-γ and TNF-α [37], as well as $CD4^+$ T cells secreting IFN-γ and IL-2 [38,39] or TNF-α [25], have been reported to correlate with

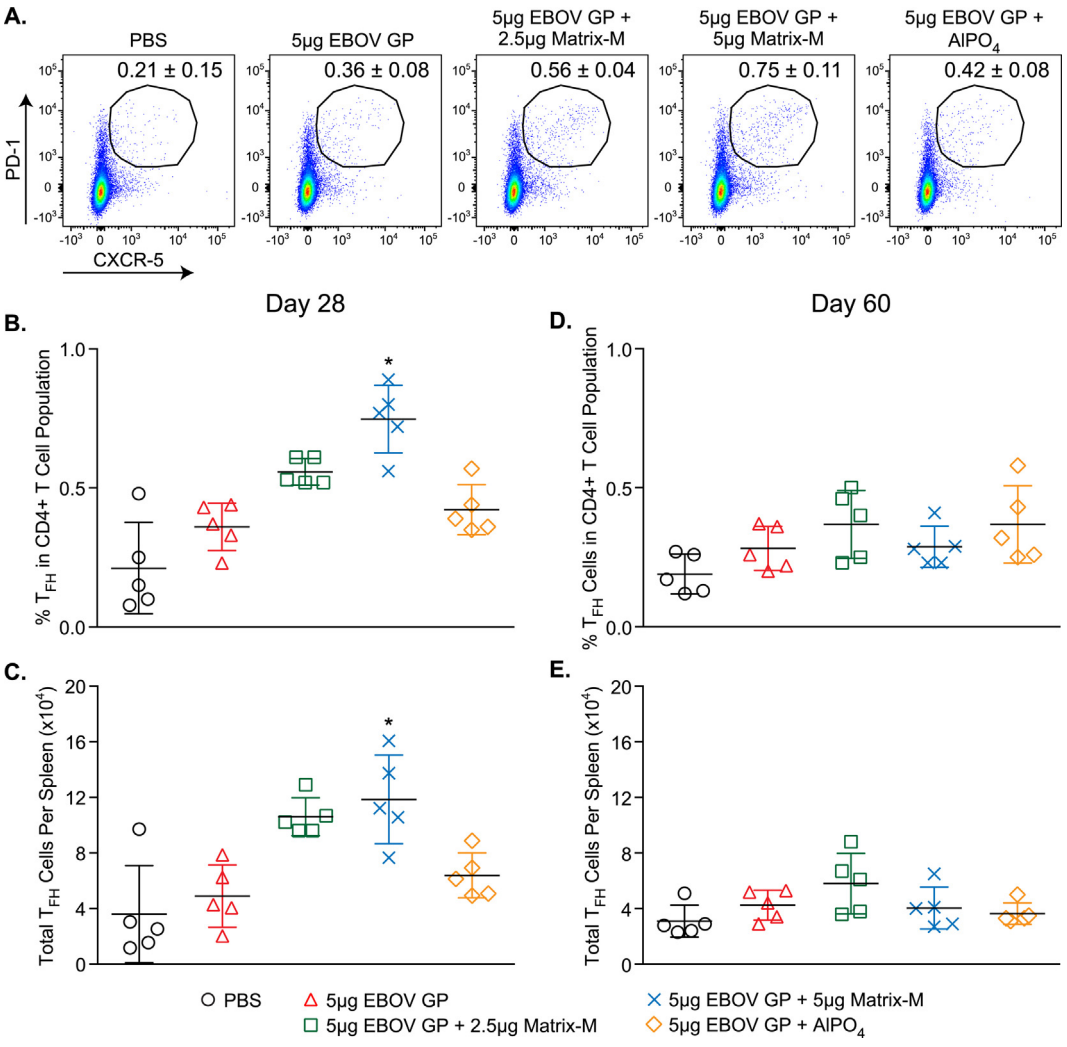

Fig. 4. Matrix-M enhanced the frequency and absolute number of T_{FH} cells in the spleen. T_{FH} cells, defined as $CXCR5^+PD\text{-}1^+$ T cells within $B220^-CD49b^-$ $CD3^+CD4^+$ T cell gate, were identified in spleens at Days 28 and 60. Representative dot-plot of T_{FH} cell analysis from each group is shown (A). The number in the dot-plot is the average frequency and standard deviation from Day 28. The frequency of T_{FH} cells within the $CD4^+$ T cell population from Days 28 (B) and 60 (D) is shown. The absolute T_{FH} cell number per spleen from Days 28 (C) and 60 (E) was calculated by multiplying the frequency of T_{FH} cells with the total number of splenocytes in the spleen. Black bars indicate group means and error bars represent standard deviation, * $p < 0.05$.

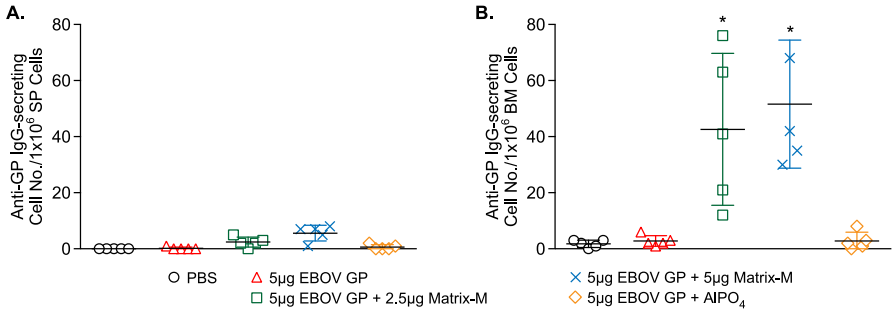

Fig. 5. Matrix-M induced persistence of EBOV/Mak GP specific plasma cells in bone marrow. Spleen and bone marrow cells were incubated overnight in EBOV/Mak GP coated ELISPOT plates. The EBOV/Mak GP-specific IgG spots were detected by incubating with goat-anti-mouse IgG-HRP followed by spot development. Spot numbers were counted and analyzed using an ELISPOT reader. The number of antibody secreting cells (ASC) per million cells is shown. (A) Day 60 EBOV/Mak GP-IgG ASC number in the spleen; (B) Day 60 EBOV/Mak GP-IgG ASC number in the bone marrow. Black bars indicate group means and error bars represent standard deviation, * $p < 0.05$.

survival. T cells that simultaneously produce two or more cytokines are defined as multifunctional T cells, and the number of multifunctional T cells is associated with a better effector activity and immunologic memory [40–42]. The use of the Matrix-M adjuvant provided a dose dependent increase in the frequency of $CD4^+$ and $CD8^+$ cytokine secreting T cells as well as the number of multifunctional T cells producing more than one cytokine. The observation that protection from a lethal EBOV challenge was observed only in the Matrix-M adjuvanted EBOV/Mak GP group was associated with the enhanced production of multifunctional T cells.

The use of Matrix-M increased the frequency of GC B cells in the spleen and EBOV/Mak GP-specific plasma cells in the bone marrow. GCs are the micro-anatomic locations for B cell differentiation, somatic hypermutation, antibody class-switching and formation of memory B cells [23,24,43]. Co-administration of the EBOV/Mak GP with Matrix-M also resulted in an increase of the numbers of T_{FH} cells which facilitate GC B cell differentiation and development [23,24,43–46]. The increased frequency of GC and T_{FH} cells induced by Matrix-M adjuvantation was associated with the enhanced magnitude of the antibody response and the induction of a greater numbers of GP-specific plasma cells in the bone marrow. This would suggest that the Matrix-M adjuvanted EBOV/Mak GP vaccine has the potential to induce a durable antibody response. Our novel observation of Matrix-M driven stimulation of GC formation and increased frequencies of T_{FH} cells, not reported before, is well in line with the typical humoral responses evoked by Matrix-M adjuvanted antigens [10,13].

In summary, our data indicates that co-administration of the Matrix-M adjuvant with purified EBOV/Mak GP nanoparticles provides robust stimulation of the anti-EBOV/Mak GP immune response resulting in 100% protective efficacy in the mouse model. We observed a more rapid onset of anti-EBOV/Mak GP IgG and EBOV neutralization antibodies, increased concentration of IgG2a, as well as increased frequency of multifunctional $CD4^+$ and $CD8^+$ T cells, T_{FH} cells, germinal center B cells and persistence of EBOV/Mak GP-specific plasma B cells in the bone marrow. These data, along with the reported safety profile of Matrix-M in clinical trials, have progressed the investigation of this vaccine into NHP studies and a phase I clinical trial.

Conflict of interest statement

KLB, HS, LS, YL, DCF, MJM, RHX, HL, TJH, EK, GG AND GS are employees of Novavax. Employees may hold stock and/or stock options in the company.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2016.02.033.

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